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Study Plan Summary
James E. Marler and J. Robert Bridges -
Alexandria, La.

DEVELOPMENT OF SELECTIVE MEDIA FOR ISOLATING MICROORGANISMS ASSOCIATED WITH THE SOUTHERN PINE BEETLE

(Cooperative Agreement No. 19- with LSUA)

Symbiotic microorganisms are known to affect the development of the southern pine beetle, *Dendroctonus frontalis* (SPB). Changes in the symbiotic microorganism complex may cause or reflect changes in SPB field populations. Studies to isolate and evaluate populations of SPB symbionts have been hampered by rapidly growing yeasts and saprophytic fungi. This study will develop media that will allow the isolation of individual SPB symbionts without interference or competition from other microorganisms.

The objectives of the study are to: 1) analyze the nutrition of the symbiotic yeasts, mycangial fungi, and *Ceratocystis minor* with the goal of using specific nutritional requirements as a basis for selection; 2) survey the available antimicrobial agents for use in selective media, and, 3) select specific agents that will allow rapid differentiation of one species from another by color, growth pattern, or chemical reaction.

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STUDY PLAN

DEVELOPMENT OF SELECTIVE MEDIA FOR ISOLATING MICROORGANISMS ASSOCIATED WITH THE SOUTHERN PINE BEETLE

James E. Marler and J. Robert Bridges
(Cooperative Agreement No. 19- with LSUA)

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INTRODUCTION

Symbiotic microorganisms significantly affect development of the southern pine beetle (SPB), *Dendroctonus frontalis* Zimm. The two mycangial fungi (#122, #133) are necessary for optimum larval growth and survival (2). The bluestain fungus, *Ceratocystis minor* (Hedgc.), inhibits larval development when it stains the phloem (1), but may otherwise benefit the beetle by creating conditions necessary for optimum brood development (6, 15, 21, 22). Many other microbes occur with the SPB (3, 4, 7, 18, 19, 20), but their roles have not yet been discovered.

Because symbiotic microorganisms influence SPB development, changes in the SPB symbiotic complex may cause or reflect changes in SPB populations. The symbiotic microorganism complex may be an important factor regulating SPB outbreaks. To test these ideas, studies are needed to correlate field populations of SPB with the composition of the symbiotic microorganism complex.

At present, these studies are impractical because we do not have methods for selectively isolating these microorganisms. For example, rapidly growing saprophytic fungi such as *Trichoderma* sps., *Penicillium* sps., or *Aspergillus* sps. often obscure the slower growing mycangial fungi and *C. minor*. Yeasts, which are found in high numbers in larval galleries, usually prevent consistent reliable isolation studies of the mycangial fungi. This study will develop media that will enable us to isolate individual symbionts without interference or competition from other microorganisms.

Previous studies on the microflora of SPB have centered on identification of the total flora. The use of selective media was primarily limited to those commercially available media (10, 16, 23) which selectively isolate either bacteria or fungi. Most of these media (mycophil, sabouraud, PDA, malt extract agar) use pH as the selective agent. Antibiotics are commonly used in these selective media to inhibit bacteria (25). Penicillin, streptomycin, chloromycetin, and gentamycin are the most common antibacterial agents and can be used to isolate yeasts and fungi free of bacteria.

Media available for the isolation of specific yeasts or fungi are limited. Pathogenic fungi can be isolated from humans using cycloheximide to inhibit other fungi (12, 14, 23). Cycloheximide has been used in selective media for *C. ulmi* (8) and other *Ceratocystis* spp. (13). Littman (16) added oxgall to a base medium to inhibit the spread of fungi. Botard (5) modified Littman's oxgall agar to isolate *Cryptococcus neoformans*. Edberg (11) used an esculin based medium for

for the isolation and color differentiation of *Cryptococcus neoformans*. *Candida* species can be differentiated by the use of BiGGY agar containing bismuth citrate and sodium sulfite (23). This medium uses the bismuth sulfite reaction as the differential agent. The fungicide, benomyl, has been used to isolate wood decay fungi from bark beetles (9). Pentachloronitrobenzene (PCNB) and o-phenyl phenol (OPP) have been used to isolate slow-growing Basidiomycetes (26). Though no specific media are commercially available for use, the possibility exists that the proper combination of selective agents can result in a useful isolation media for SPB yeasts, mycangial fungi, and *C. minor*.

STUDY OBJECTIVE

This study has an objective of developing a method of isolating and differentiating SPB yeast and mycangial fungi, using selective and/or differential growth media. This objective will have three phases: 1) to completely analyze the nutrition of the symbiotic yeasts, mycangial fungi, and *C. minor* with the goal of using specific nutritional requirements as a basis for selection; 2) a survey of available antimicrobial agents for use in selective media; 3) and, to use specific agents that will allow differentiation of one species from another by color, growth pattern, or chemical reaction.

MATERIALS AND METHODS

In the recent taxonomic studies on yeasts of the SPB using Wickerham's (27) method for carbon assimilation, we found that several carbon sources promoted the growth of selected species of yeast. Therefore, we will use the same auxanographic plate method on hyphal SPB fungi to determine which sugars or other carbon sources promote the growth of each species to be isolated. Sugar and other carbon sources (10% w/v) will be filter sterilized and placed on sterile 1/4" paper disc (Difco Sterile Blanks #1599-35). After oven drying at 50°C, these discs will be placed on inoculated plates for observation of growth around each carbon source. A table of genus and/or species specific carbon sources will be compiled for use in future selective media.

The screening of antimicrobial compounds will be done in the following manner: 1) for *C. minor* and mycangial fungi plates of media containing the antimicrobial compound will be inoculated with 5 mm agar plugs of the fungi. Radial growth of fungi will be measured and compared to the growth on control plates; 2) the unicellular nature of yeast will allow us to test them as we would bacteria. Various species of SPB yeast will be streaked and/or spread plated on the antimicrobial test medium. Qualitative evaluations will be based on relative inhibition after incubation for 5 days at 30°C. For quantitative tests the tube dilution method will be used to determine the

minimum inhibitory concentration (MIC) for each yeast studied (24). 3) two or more organisms will be mixed together, gently homogenized to fragment large filaments and clumps, and spread plated on the "selective" medium. Qualitative evaluations will be made on the action of the antimicrobial compound on mixed cultures and on the recovery of the desired organism or organisms. Among the agents being considered for study are: griseofulvin, nystatin, potassium iodide, miconazole, clotrimazole, primaricin and tolnafate, cadmium succinate, PCNB, $CuSO_4$, dicloran, mancozeb zineb, dodine, anilazine, thiram, benomyl, thiabendazole, thiophanate, and OPP.

The last aspect will be the inclusion of compounds or chemical tests to show species differences. Bismuth salts, esculin, TTC, and other color differential compounds will be used in the tests because they have already been reported in the literature as being useful. Combinations of these compounds will be tested to develop a broad spectrum differential medium. Color and colony growth differences are often subject to environmental variables so pH, temperature, and incubation time will be standardized for each type of medium developed.

STATISTICAL ANALYSIS OF THE DATA

This study will develop media to inhibit certain microorganisms while allowing others to grow. Much of the screening of various media can be done by observing the relative growth of the microorganisms and will not require statistical analysis.

To quantitatively evaluate media for the hyphal fungi, radial growth of fungi on test media will be compared to growth on a control medium using an analysis of variance. For yeasts, the methods of Shadomy and Espinel-Ingroff (24) will be used to determine the minimum inhibitory concentration of the antimicrobial compounds.

DATES OF ESTABLISHMENT AND COMPLETION

Work on this study will begin on May 1, 1981 and will be completed by Dec. 31, 1982.

FINANCIAL PLAN

A. Forest Service share of cost

1. Salary and wages

a. Principal investigator 100% of time, 3 months in summer of 1981	$2750
b. Research Assistant 100% of time, 3 months in summer of 1981	2750
c. Supplies and miscellaneous services	500
TOTAL Forest Service Share	$6000

B. University Share of cost

1. Salaries
   a. Principal investigator, 15% of time
   b. Research assistant, 15% of time
2. Employee benefits
3. Indirect costs, 40% of salary and wages
   Ref. FMC 73-6 Negotiation 7-24-77

TOTAL University Share

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Peter L. Lorio, Jr.
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Appropriation, limitation, or project symbol	Appropriation title	Limit'n. or Proj't. Amount	Appropriation Amount

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Campus Correspondence

LOUISIANA STATE UNIVERSITY
AT ALEXANDRIA

Rockwell

From: Dr. J. E. Marler
Professor of Biology - Division of Science
LSU-A

Date: October 1, 1981

To: Southern Forest Experiment Station

Re: Progress Report No. 19-288

The project was funded and began June 1st, 1981.

A three phase study was initiated as follows:

(1) Analyze the nutrition of SPB yeast, Ceratocystis minor, and mycangial fungi #122 and 133. We hope to find specific nutritional requirements that might be a basis for selective or differential media.

(2) We obtained a large number of microbial inhibitors to use in the development of a selective medium.

(3) We obtained several specific differential agents that are commonly used in bacterialogical media with the idea of adopting them to mycological media. Thus we would be able to rapidly differentiate one group of microbes from another by color, growth pattern, and/or chemical reaction.

I. Nutritional Study

Basic media being used in the nutritional study:

(1) Sabadraud dextrose Agar (Difco) our code - SAB

(2) Potatoe dextrose Agar (Difco) our code - PDA

(3) Snyders test Agar (Difco) our code - BCG

(4) Carbon Assimilation Agar (Difco) our code - YAM

These media were analyzed for usefulness in the three phase study outlined previously. Traditionally this lab has used various formulations of Malt extract agar for routine growth of yeast and hyphal fungi. From experience it became evident that several problems existed in the use of ME agar:

A. Many yeast gave the same cultural characteristics even though there may be two or more genera present.

B. Mycangial fungi grew at different rates on ME. C. minor would overgrow the whole plate in 48-72 hours. 133 grew moderately well, taking 5-7 days to reach >60 cms in diameter. However, 122 grew very slowly, requiring 2 weeks or more to reach relatively large size. While C. minor and 133 grew well at 30°C, 122 required 25°C incubation for growth on ME. Thus any plate with C. minor on it was "wall to wall" before 133 or 122 could be differentiated.

C. Different formulations (2.5%, 1.5% ME) were routinely stocked to grow 133 or 122.

D. "Wild" fungi, i.e. Trichoderma, Aspergillus, Penicillium grew too well on M.E., usually overgrowing the plate before 133 or 122 can be identified.

With these "problems" in mind, several other common mycological media were tried to substitute for M.E., PDA and SAB and various formulation of each proved to be less productive in growing the mycangial fungi #133 and 122 than ME so a basal salts solution was used to develop an understanding of the nutritional need of the fungi, especially 122.

The basal salt medium was bacto-carbon assimilation medium (Difco Co.) used for yeast in previous studies. We called this medium "yeast assimilation media" or Y.A.M. for short. We choose this medium because of the large number of yeast also encountered with pine bark beetles and the success of this medium in growing these yeast.

Ammonium Sulfate was the N source in YAM but it proved unsatisfactory for our hyphal fungi so an organic nitrogen source-CAS-Amino Acids-was used. We surveyed the following carbohydrates: Dextrose, Fructose, Calactose, Maltose, and Starch. The initial survey showed that Maltose was the best of these carbohydrates.

YAM is rather expensive to buy and tedious to make by hand. We went back to ME and added CAS Amino Acids + Maltose for a formulation as follows: Super Malt Extract Medium

1% Malt Extract
1% Yeast Extract (or a Vitamin Mix)
1% CAS Amino Acids (+ .1% tryptophane)
1% Maltose
2% Agar (if required)

This proved superior to ME alone for overall growth of Mycangial Fungi. However, the nutritionally rich nature of "Super Malt extract" (or SME) resulted in less spread and thicker vertical growth. C. Minor and 133 produced few spores and no perithecia. 122 grew as well as 133 and no longer required 25° incubation, growing at 30° C as well as or better than at 25° C. The typical brown 122 color developed much slower on SME, starting in the center and slowly spreading outward as the mycelium ages. This typical brown color may take up to 10 days or more to develop.

We are continuing the study of carbohydrate assimulation of pine beetle fungi with detailed growth rate studies to follow.

Liquid culture growth of C. Minor, 133 and 122 became routine using SME. C. Minor and 133 grew as dimorphic fungi--hyphal phase on agar plates and primarily a yeast phase in liquid culture. The 122 fungus grew as small (<1 mm) to medium (1-3 mm) balls with no yeast phase.

Preliminary studies into the pH range of C. Minor, 133 and 122 were started and liquid culture results were encouraging. A detailed study of pH range (3-7) is underway using both liquid cultures and agar plates.

Temperature range studies are being contemplated for the next physical growth requirement investigation.

II. Antimicrobial Study

The following microbial inhibitors were tested on C. Minor, 133, and various yeast species:

Benlate: Methyl-1- (butyl carbamyl)-2-benzimidazol carbamate

Captan: Cis-N-(trichloromethyl) thio)-4-cyclohexene-1,2 dicarboximide

Clotrimazole: 1-(O-chloro-α,α-diphenylbenzyl) imidazole

Crystal violet: gentian violet

Cycloheximide: 1, 2, 3, 4-tetrahydrobenzene

Criseofulvin: Sporostatin (TM)

Miconazole: Dermonistat (TM)

Oxgall (DIFCO): Sodium salts of glycocholic and Taurocholic acid

PCNB (Terraclor (TM): Pentachloronitrobenzene

Rosebengal (Acid Red)

Chapman's Tellurite: Potassium tellurium dioxide (1%)

Tolnaftate: Tinactin (TM)

Zinab: Zinc ethelene-bis-dithiocarbamate

Visual observations (mycelial diameters, etc.) will be reported in future progress reports. Other antimicrobials may be added to the above list as they are obtained.

Generally speaking, no antifungal compound was found that inhibited yeast only or hyphal fungi only, or all microbes but pine beetle fungi. However, one compound used in commercial selective mycological media was

found to be useful in our pine beetle microbe studies. This was oxgall (Difco). This compound prevents the rapid spreading of certain fungi but does not inhibit mycelial development or yeast growth. We plan to use this as part of future differential media.

III. Differential Compound Study

Several differential compounds commonly used in bacterial media were tested for usefulness:

(1) Brom cresol Green: From BCG Agar

(2) TTC: From Enterococcus Agar

TTC gave light pink to deep maroon color to yeast and fungi colonies. The color differentiation was interesting and may have some application. Some of the yeast were inhibited by increased conc. of TTC but the hyphal fungi showed no inhibition.

Brom Cresol Green (BCG) was non inhibitory and gave various colony colors. We will use this dye to differentiate yeast genera during pine beetle studies of yeast. Hyphal fungii (CM, 133, 122) grew as pale green mycelium and gave no color differentiation, so BCG has questionable application with these organisms.

Extensive work on a differential medium will continue.